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Author

Grasis, Juris A

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Chapter 1

Host-Associated Bacteriophage Isolation and Preparation for Viral Metagenomics

Juris A. Grasis

Abstract

Prokaryotic viruses, or bacteriophages, are viruses that infect bacteria and archaea. These viruses have been known to associate with host systems for decades, yet only recently have their influence on the regulation of host-associated bacteria been appreciated. These studies have been conducted in many host systems, from the base of animal life in the Cnidarian phylum to mammals. These prokaryotic viruses are useful for regulating the number of bacteria in a host ecosystem and for regulating the strains of bacteria useful for the microbiome. These viruses are likely selected by the host to maintain bacterial populations. Viral metagenomics allows researchers to profile the communities of viruses associating with animal hosts, and importantly helps to determine the functional role these viruses play. Further, viral metagenomics show the sphere of viral involvement in gene flow and gene shuffling in an ever-changing host environment. The influence of prokaryotic viruses could, therefore, have a clear impact on host health.

Key words Metagenomics, Viral metagenomes, Virome, Microbiome, Prokaryotic virus, Bacteriophage, Host-microbe interactions, Holobiont, Symbiosis

1 Introduction

Prokaryotic viruses (commonly known by the anachronistic term bacteriophage, *see* **Note 1**) infect bacteria and archaea. There are two modes of the prokaryotic viral life cycle, lytic and temperate. The lytic phase involves the infection, replication, and lysis of the bacterium, leading to the death of the cell and escape of viral progeny. The temperate phase involves the integration of the prokaryotic virus into the genome of the bacterium in a proviral form, which when activated at a later time can then become a lytic virus.

The involvement of prokaryotic viruses in host-associated regulation of the microbiome has only recently been appreciated. These viruses likely help to regulate the number of bacteria and the strains of bacteria associating with a host. These associations have an impact on host metabolism [1], immunity [2], as well as animal health and disease [3]. To best evaluate the effects these viruses

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have on host ecosystems, it is useful to use viral metagenomics to fully assess which genes in the viral genomes have these effects on the microbiome. This protocol will allow the researcher to isolate, purify, and prepare viral nucleic acid for sequencing.

It is important to purify viruses because the total amount of viral nucleic acid is small compared to that of bacterial and host nucleic acid content [4]. The amount of nucleic acid per virus is estimated to be approximately 1 attogram (10^{-18} g). Most current sequencing platforms require at least 1 nanogram (10^{-9} g) of DNA. Therefore, if you have less than 10^9 viruses in your sample, you will need to enrich the viruses and amplify the DNA. Physical enrichment of viruses increases the number of viral sequences. Increased number of viral sequences means greater representation in databases, which will increase the likelihood of database matches. Further, enrichment of viruses for sequencing is necessary, as less than 5% of the unenriched metagenome sequences will contain viral sequences. The most effective purification method is a three-step technique utilizing centrifugation, filtration, and nuclease treatment. Using this technique eliminates more than 80% of host/bacterial contamination as compared to without purification, while using additional purification methods (e.g., density gradients) selects for certain populations of viruses. Similarly, use of viral DNA/RNA extraction kits is not recommended, as they create biases in viral populations [5]. Random shotgun sequencing libraries are recommended, as one can amplify a sample using barcoding primers. Even if you have enough DNA, it is recommended to exercise this protocol to barcode your samples for sequencing. It is not recommended to use multiple displacement amplification for amplifying viral DNA, as the Phi29 polymerase preferentially amplifies circular and single-stranded DNA and can, therefore, amplify contaminating host/bacterial DNA as well as small, circular viral DNA, which can alter the viral community profile prior to sequencing.

The use of a CsCl density gradient for purification of bacteriophages is up to the user. CsCl density gradients are effective at removing host and bacterial DNA, leaving more viruses for sequencing. This comes at a cost, as these gradients select for certain viruses while discriminating against others according to viral specific density. Further, reproducibility of CsCl density gradients is an issue [6]. It is therefore recommended to avoid using CsCl density gradients if accurate viral populations and reproducibility are of concern. If host and bacterial contaminations are of concern, this section is provided as an option. Also, an ultracentrifuge is needed for this optional step. Alternatively, one can repeat nuclease treatment prior to viral nucleic acid purification.

Although only two families of RNA bacteriophages have been described to date, this does not mean that the number and the diversity of RNA bacteriophages are less than those of DNA

bacteriophages. More likely, the large percentage of DNA bacteriophages found in databases is due to the purification and selection method used (e.g., CsCl gradient selection) and consequent preferential sequencing of these selected viruses. Further, working with RNA samples is difficult due to the labile nature of RNA and the ubiquitous presence of RNases. Therefore, it is recommended to split the samples into DNA and RNA fractions to sample both DNA and RNA bacteriophages. Make sure that the working space, equipment, and reagents are as RNase free as possible. Keep in mind that the low number of characterized RNA bacteriophages will yield low numbers of hits in databases. However, as researchers sequence more RNA bacteriophages, more of these viruses will be characterized, and the RNA bacteriophage database will increase. Therefore, it is recommended to sequence both DNA and RNA bacteriophages.

This protocol takes the user from the isolation of bacteriophages (and other viruses, *see* **Note 2**) in host-associated environments, purification of bacteriophages, enumeration of viral-like particles (VLPs) through epifluorescence microscopy [7], characterization of VLPs through transmission electron microscopy [8, 9], extraction of viral DNA (vDNA) [10] and viral RNA (vRNA) [11, 12], and barcoding and amplification of viral nucleic acids for sequencing using Illumina sequencing technologies.

2 Materials

2.1 Extraction of Bacteriophages from Host Tissue

1. Molecular grade dH_2O (sterile, nuclease-free, virus-free dH_2O).
2. Saline magnesium (SM) buffer, pH 7.5, per 100 mL: To 80 mL molecular grade dH_2O add 0.58 g NaCl (100 mM), 5 mL 1 M Tris–HCl pH 7.4 (50 mM), 0.25 g $MgSO_4$ $7H_2O$ (10 mM), 0.1 g gelatin (0.1%, optional), adjust to pH 7.4, fill to 100 mL with molecular grade dH_2O, autoclave to sterilize, store at room temperature.
3. Microcentrifuge pestle (cleaned with 70% ethanol and RNA-Zap).
4. Handheld electric tissue homogenizer (cleaned with 70% ethanol and RNA-Zap).
5. 5 mL Sterile syringes.
6. 0.45 μm Syringe filters, PVDF, 28 mm diameter, sterile.
7. 0.02 μm Syringe filters, 25 mm diameter, sterile (Whatman Anotop-25).
8. Chloroform, use in a chemical hood.

9. DNase I (10 U/μL) and 10× DNase buffer (100 mM Tris pH 7.5, 5 mM $CaCl_2$, 25 mM $MgCl_2$).
10. RNase I (10 U/μL).
11. 4% Paraformaldehyde (PFA), per 10 mL: To 10 mL molecular grade dH_2O add 0.4 g PFA, heat at 50 °C for 1 h, fill to 10 mL with molecular grade dH_2O, 0.02 μm filter sterilize, good for 1 month at 4 °C.
12. 5% Formaldehyde (FA), per 10 mL: To 8 mL molecular grade dH_2O add 0.5 g formaldehyde, mix, and fill to 10 mL with molecular grade dH_2O, 0.02 μm filter sterilize, good for 1 month at 4 °C.

2.2 Purification of Bacteriophages (Optional)

1. Cesium chloride (CsCl) density step gradient—made in buffer used for resuspension of sample.
   (a) 1.7 g/mL (w/v): To 7.5 mL of resuspension buffer add 9.5 g CsCl, weigh 1 mL to adjust to final concentration of 1.7 g/mL with buffer or CsCl.
   (b) 1.5 g/mL (w/v): To 8.2 mL of resuspension buffer add 6.7 g CsCl, weigh 1 mL to adjust to final concentration of 1.5 g/mL with buffer or CsCl.
   (c) 1.35 g/mL (w/v): To 9.9 mL of resuspension buffer add 5.0 g CsCl, weigh 1 mL to adjust to final concentration of 1.35 g/mL with buffer or CsCl.
   (d) 1.2 g/mL (w/v): To 9.9 mL of resuspension buffer add 2.4 g CsCl, weigh 1 mL to adjust to final concentration of 1.2 g/mL with buffer or CsCl.
2. Ultracentrifuge tubes (Ultraclear 14 × 89 mm, Beckman Coulter).
3. SW41 Ti swinging bucket ultracentrifuge rotor.
4. Ultracentrifuge (Beckman Coulter).
5. 18-gauge needles.
6. 5 mL Syringes.

2.3 Viral-Like Particle Counts Using Epifluorescent Microscopy

1. Molecular grade dH_2O (sterile, nuclease-free, virus-free dH_2O).
2. 0.02 μm Anodisc.
3. SYBR Gold (10,000×).
4. Mounting solution, 0.1% ascorbic acid, 50% glycerol. Per 10 mL: To 4.9 mL molecular grade dH_2O add 100 μL of 10% ascorbic acid, then slowly add 5 mL glycerol, mix thoroughly, divide into 1 mL aliquots, and store at −20 °C.
5. Vacuum pump.
6. Microscopy slides.

7. Microscopy coverslips.
8. Forceps.
9. Petri dish.
10. Epifluorescent microscope with standard objective orientation (63× objective).

2.4 Transmission Electron Microscopy of Viral-Like Particles

1. Molecular grade dH_2O (sterile, nuclease-free, virus-free dH_2O).
2. 2% Uranyl acetate, pH to 4.5. Per 10 mL: To 9 mL molecular grade dH_2O add 0.2 g uranyl acetate, adjust pH to 4.5 with NaOH, and fill to 10 mL with molecular grade dH_2O; this chemical should be handled with care since it is both toxic and slightly radioactive [8, 9], and stored at 4 °C for up to 2 years.
3. Electron microscopy grids, 200–400 square mesh, copper, stabilized with a 2–10 nm thick carbon-layer Formvar film: Grids can become hydrophobic over time and may show poor adsorption of bacteriophages; therefore, it is recommended to treat the grids with UV light or poly-L-lysine to make the grids hydrophilic again.
4. Parafilm.
5. Forceps.
6. Filter paper.
7. Transmission electron microscope.

2.5 Viral DNA Extraction

1. 0.5 M EDTA, pH 8.0: Per 10 mL: To 8 mL molecular grade dH_2O add 1.86 g EDTA, adjust pH to 8.0 with NaOH (~200 μL), fill to 10 mL with molecular grade dH_2O, sterilize through 0.02 μm filter, and store at room temperature.
2. 2 M Tris–HCl, 0.2 M EDTA, pH 8.5: Per 10 mL: To 3 mL molecular grade dH_2O add 2.4 g Tris, 4 mL 0.5 M EDTA pH 8.0, adjust pH to 8.5 with HCl, fill to 10 mL with molecular grade dH_2O, sterilize through 0.02 μm filter, and store at room temperature.
3. Formamide (10 mL): Store at 4 °C.
4. Glycogen (20 mg/mL): Store at −20 °C.
5. 100% Ethanol (100 mL): Store at room temperature.
6. 1 M Tris–HCl pH 8.0: Per 10 mL: To 8 mL molecular grade dH_2O add 1.21 g Tris, adjust to pH 8.0, fill to 10 mL with molecular grade dH_2O, sterilize through 0.02 μm filter, and store at room temperature.
7. 10 mM Tris, 1 mM EDTA (TE), pH 8.0: Per 100 mL: To 80 mL molecular grade dH_2O add 1 mL 1 M Tris–HCl pH 8.0, add 2 mL 0.5 M EDTA, adjust pH to 8.0, fill to 100 mL with

molecular grade dH_2O, sterilize through 0.02 μm filter, and store at room temperature.

8. 10% Sodium dodecyl sulfate (SDS): Per 10 mL: To 10 mL molecular grade dH_2O add 1 g SDS, store at room temperature.
9. Proteinase K (20 mg/mL): Store at −20 °C.
10. 5 M NaCl: Per 10 mL: To 10 mL molecular grade dH_2O add 2.92 g NaCl, sterilize through 0.02 μm filter, and store at room temperature.
11. 10% Cetyltrimethyl ammonium bromide (CTAB), 700 mM NaCl: Per 10 mL: To 6 mL molecular grade dH_2O add 1 g CTAB, add 0.4 g NaCl, heat to 65 °C to dissolve CTAB/NaCl, fill to 10 mL with molecular grade dH_2O, store at room temperature, and preheat to 65 °C before use.
12. Chloroform (100 mL): Keep in glass container, store at room temperature, and use in a chemical hood.
13. Phenol:chloroform:isoamyl alcohol (25:24:1), pH 8.0 (100 mL): Keep in glass container, store at 4 °C, and use in a chemical hood.
14. Isopropanol (100 mL): Store at room temperature.
15. 70% Ethanol (100 mL).
16. Molecular grade dH_2O (sterile, nuclease-free, virus-free dH_2O).

2.6 Viral RNA Extraction

1. RNase-ZAP (Thermo Fisher Scientific) or 1% SDS: Per 100 mL: To 100 mL molecular grade dH_2O add 1 g SDS, store at room temperature.
2. Guanidine isothiocyanate RNA lysis buffer (GITC buffer, TRIzol LS, Thermo Fisher Scientific) (100 mL): Store at 4 °C.
3. 10 mM Dithiothreitol (DTT): Store at −20 °C.
4. Chloroform (100 mL): Keep in a glass container, store at room temperature, and use in a chemical hood.
5. Isopropanol (100 mL): Store at room temperature.
6. Glycogen (20 mg/mL): Store at −20 °C.
7. RNase-free 70% ethanol (100 mL): Use RNase-free molecular grade dH_2O to make 70% ethanol.
8. Molecular grade dH_2O (sterile, nuclease-free, virus-free dH_2O).

2.7 Quality Control to Determine Bacterial/Host Contamination

1. 10× *Taq* polymerase buffer: 100 mM Tris–HCl pH 8.4, 500 mM KCl, store at −20 °C.
2. BSA (1 mg/mL): Store at −20 °C.
3. 10 mM $MgCl_2$: Store at −20 °C.

4. 10 mM dNTPs: Store at −20 °C.
5. *Taq* polymerase (10 U/μL): Store at −20 °C.
6. Molecular grade dH_2O (sterile, nuclease-free, virus-free dH_2O).
7. Prokaryotic primers: Store at −20 °C:
   (a) 1 mM Eub27F—20-mer: 5′—AGR GTT TGA TCM TGG CTC AG—3′.
   (b) 1 mM Eub1492R—19-mer: 5′—GGH TAC CTT GTT ACG ACT T—3′.
8. Eukaryotic primers: Store at −20 °C:
   (a) 1 mM EukF—21-mer: 5′—AAC CTG GTT GAT CCT GCC AGT—3′.
   (b) 1 mM EukR—24-mer: 5′—TGA TCC TTC TGC AGG TTC ACC TAC—3′.

2.8 Viral RNA First-Strand Synthesis

1. Reverse Transcriptase (200 U/μL, SuperScript III, Thermo Fisher Scientific).
2. 5× Reverse transcriptase reaction buffer: 250 mM Tris–HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$, store at −20 °C.
3. 10 mM dNTPs: Store at −20 °C.
4. 10 μM Random hexamer primer (N6): Store at −20 °C: 5′—NNN NNN—3′.
5. 50 nM Anchored oligo dT primer (dT18a): Store at −20 °C: 5′—TTT TTT TTT TTT TTT TTT VN—3′.
6. 10 mM Dithiothreitol (DTT): Store at −20 °C.
7. RNase inhibitor (40 U/μL): Store at −20 °C.
8. 10 mM $MgCl_2$: Store at −20 °C.
9. 1 M Dimethyl sulfoxide (DMSO): Store at −20 °C.

2.9 Viral Second-Strand Synthesis

1. Molecular grade dH_2O (sterile, nuclease-free, virus-free dH_2O).
2. T4 DNA polymerase (5 U/μL).
3. 10× T4 DNA polymerase buffer: 250 mM Tris-acetate pH 7.5, 1 M potassium acetate, 100 mM magnesium acetate, and 5 mM DTT, store at −20 °C.
4. 10 mM dUTPs: Store at −20 °C.
5. RNase H (250 U/μL): Store at −20 °C.
6. 10 mM DTT: Store at −20 °C.

2.10 DNA Cleanup

1. SPRI Beads (Agencourt AMPure XP Beads, Beckman Coulter): Store at 4 °C. Bring to room temperature before use.
2. Magnetic tube rack.

3. 70% Ethanol (70% EtOH).
4. Molecular grade dH_2O (sterile, nuclease-free, virus-free dH_2O).

2.11 DNA Shearing

1. Covaris shearing microtubes (microTUBE-50).
2. Covaris focused ultrasonicator (M220).

2.12 DNA End Repair

1. T4 DNA polymerase (5 U/μL).
2. 10× T4 DNA ligase reaction buffer: 500 mM Tris–HCl pH 7.5, 100 mM $MgCl_2$, 10 mM ATP, and 100 mM DTT, store at −20 °C.
3. T4 polynucleotide kinase (10 U/μL): Store at −20 °C.
4. 10 mM dNTPs: Store at −20 °C.
5. Molecular grade dH_2O (sterile, nuclease-free, virus-free dH_2O).

2.13 Adenylate 3′ Ends

1. DNA polymerase (5 U/μL), Klenow Fragment (3′–5′ exo-).
2. 10× Klenow reaction buffer: 500 mM NaCl, 100 mM Tris–HCl pH 7.9, 100 mM $MgCl_2$, 10 mM DTT, store at −20 °C.
3. 10 mM dATP: Store at −20 °C.
4. Molecular grade dH_2O (sterile, nuclease-free, virus-free dH_2O).

2.14 Adapter Ligation

1. Annealed adapters (see Section 2.18).
2. T4 DNA ligase (20 U/μL).
3. T4 DNA ligase reaction buffer: 500 mM Tris–HCl pH 7.5, 100 mM $MgCl_2$, 10 mM ATP, and 100 mM DTT, store at −20 °C.

2.15 Uridine Removal for Viral RNA Samples (Skip for Viral DNA Samples)

1. Uracil-DNA glycosylase (1 U/μL, UDG).
2. 10× UDG reaction buffer: 200 mM Tris–HCl, 10 mM DTT, 10 mM EDTA, pH 8.0, store at −20 °C.

2.16 Size Selection of Adapter-Ligated Fragments

1. SPRI Beads: Store at 4 °C, bring to room temperature before use.
2. Magnetic tube rack.
3. 70% Ethanol (70% EtOH).
4. Molecular grade dH_2O (sterile, nuclease-free, virus-free dH_2O).

2.17 Large-Scale PCR of Size-Selected Adapter-Ligated Fragments

1. Molecular grade dH_2O (sterile, nuclease-free, virus-free dH_2O).
2. High-Fidelity DNA Polymerase (2 U/μL, NEB Q5).
3. 5× High-Fidelity DNA polymerase reaction buffer: Store at −20 °C.
4. 10 mM dNTPs: Store at −20 °C.

5. 1 mM PCR Primer 1 (Illumina TruSeq P5): Store at −20 °C: 5′- AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GA—3′.
6. 1 mM PCR Primer 2 (Illumina TruSeq P7): Store at −20 °C: 5′- CAA GCA GAA GAC GGC ATA CGA GAT—3′.

2.18 Preparation of Adapters and Indices

1. Purchase Universal Adapter and Indexed Adapters: Universal adapter must have a 3′ phosphorothioate bond and indexed adapters must have 5′ end phosphorylated: universal adapter (Illumina TruSeq): 5′- AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC*T—3′ {* = Phosphorothioate bond} and indexed adapters (Illumina TruSeq): 5′- P—GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CAC—NNN NNN—ATC TCG TAT GCC GTC TTC TGC TTG—3′ {5′ end needs to be phosphorylated, Ns are barcode}. Note that the latest Illumina TruSeq adapters are pre-annealed, so these steps may not be necessary. Check the Illumina TruSeq manual to make sure. The following steps are listed in the event the adapters are not pre-annealed.
   (a) Resuspend each adapter in molecular grade dH_2O at 570 μM (volume for 100 μM/5.7, store at −80 °C).
   (b) For annealing, make a working dilution of 10 μM by diluting 5 μL of 570 μM stock with 280 μL TE (store at −20 °C).
   (c) Mix equal volumes of the universal adapter (10 μM) with each of the indexed adapters (10 μM).
   (d) Either boil the mixes for 2 min and then cool slowly to room temperature—OR—use thermocycler program set to 1 cycle (Adapt_Anneal Program):
      - 95 °C for 10 min
      - 72 °C for 5 min
      - 60 °C for 5 min
      - 50 °C for 5 min
      - 40 °C for 5 min
      - 30 °C for 5 min
      - 20 °C for 5 min
      - 10 °C for 5 min
      - 4 °C Hold
   (e) Store the annealed adapters at −20 °C.

3 Methods

Carry out all procedures at 4 °C or on ice unless otherwise specified.

3.1 Extraction of Bacteriophages from Host Tissue

1. Resuspend sample in equal volume of molecular grade dH_2O or SM buffer (*see* **Note 3**) as volume of tissue in appropriate sized tube, e.g., microcentrifuge tube, Falcon tube.
2. Homogenize sample with handheld homogenizer at 4500 × *g* for 60 s or use microcentrifuge mortar and pestle until tissue becomes a slurry.
3. Centrifuge at slow speed (~2500 × *g*) for 20 min at 4 °C to pellet debris (eukaryotic and prokaryotic cells).
4. Optional viral precipitation step (if there is a lot of volume (> 5 mL), *see* **Note 4**). Add 10% (w/v) polyethylene glycol (PEG) 8000 and 1 M (final concentration) NaCl to sample and dissolve. Incubate overnight at 4 °C. Centrifuge to pellet at 4500 × *g* for 20 min at 4 °C. Resuspend pellet in 1 mL of same buffer used as before. Centrifuge at 4500 × *g* for 5 min at 4 °C.
5. Transfer supernatant to a new microcentrifuge tube.
6. Pre-wet 0.45 μm filter with 100 μL molecular grade dH_2O or SM buffer.
7. Filter supernatant through 0.45 μm filter to further remove unwanted debris and cells. Expect some volume loss due to filter retention.
8. Optional chloroform treatment step (*see* **Note 5**):
   (a) Add 0.2 volumes chloroform and mix by inversion or vortex to lyse any cells that have made it through filtration.
   (b) Incubate at 4 °C for 30 min, vortexing every 5 min.
   (c) Centrifuge at 4500 × *g* for 10 min at 4 ° C to pellet chloroform.
9. Add DNase buffer to filtered sample to 1× concentration.
10. Add DNase (final concentration 1 U/mL) (*see* **Note 6**).
11. Optional RNase treatment (final concentration 1 U/mL) (*see* **Note 7**).
12. Incubate for 2 h at 37 °C or overnight at room temperature.
13. Heat-inactivate DNase for 20 min at 65 °C (*see* **Note 8**).
14. Sample for epifluorescent microscopy: in a separate microcentrifuge tube transfer 15 μL sample and add 5 μL 4% PFA for a final concentration of 1% PFA.
15. Save 20 μL sample for electron microscopy. Hold at 4 °C until processed for electron microscopy. Add 5 μL 5% formaldehyde

for a final concentration of 1% formaldehyde to fix sample if not processed for a day or longer.

16. Use remaining ~1.0 mL for nucleic acid extraction and continue immediately to convenient stopping points. Split sample in half for viral DNA (vDNA) extraction (Section 3.5) and viral RNA (vRNA) extraction (Section 3.6).

3.2 Purification of Bacteriophages (Optional) [13]

1. For an overlay density step gradient, lay 1 mL of each step in the centrifuge tube starting with the highest density step (1.7 g/mL) and ending with the lowest density step (1.2 mg/mL). Be careful not to mix any of the steps while preparing the gradient.
2. Mark each layer with an indelible pen to note each layer.
3. Overlay the sample on top of the gradient, and fill to the top using the same buffer used for resuspension of the sample. Be careful not to disrupt the gradient.
4. Carefully balance each tube against each other to ensure that the mass is equivalent (<0.001 g difference between tubes); use resuspension buffer to balance the tubes.
5. Ultracentrifuge at ~82,000 × *g* using SW41 Ti rotor for 2 h at 4 °C; use a slow acceleration and no brake for the deceleration.
6. Carefully remove each tube from the rotor, making sure not to disrupt the gradient.
7. Using an 18-gauge needle, pierce the tube at the 1.5 g/mL layer mark and extract 1.5 mL of the 1.5 g/mL to 1.35 g/mL layer and interface (*see* **Note 9**).
8. Split the sample for vDNA and vRNA extractions (Sections 3.5 and 3.6).

3.3 Viral-Like Particle Counts Using Epifluorescent Microscopy (See Note 10) [7]

1. Dilute 10 μL fixed viral sample into 5 mL molecular grade dH_2O (1:500 dilution).
2. Filter sample onto 0.02 μm Anodisc filter under vacuum pressure of 10 mm Hg (10 psi or ~60 kPa). Filter is unidirectional, so be sure to have the shiny ring side up towards the sample.
3. Remove the filter tower and the filter while still under vacuum pressure.
4. Pipette 100 μL freshly made 1–5× SYBR Gold (to 995 μL molecular grade dH_2O add 5 μL10,000× SYBR Gold (50×, which can be stored at −20 °C for 1 month), then do another 1:10 dilution with molecular grade dH_2O to make 5× SYBR Gold) solution onto a Petri dish, and place the filter sample side up on the droplet to stain for 10 min at room temperature in the dark.

5. Using forceps, lift filter and place on another 100 µL droplet of molecular grade dH_2O on the Petri dish to wash the filter for 10 min at room temperature in the dark.
6. Pipette 10 µL of mounting solution onto a clean microscope slide to hold the filter in place.
7. Place stained and washed filter sample side up on microscope slide.
8. Add 10 µL of mounting solution on top of the filter and cover the filter with a coverslip. Be sure not to leave any air bubbles between the filter and the coverslip.
9. Count bacterial cells and viral-like particles (VLPs) under 485 nm light excitation using standard-orientation epifluorescence microscopy.
10. Count and document at least ten fields per slide. Average the number of VLPs and multiply by dilution factor and field/objective factor to obtain VLPs/mL.
11. Slides can be stored at −20 °C in a light-protected container.

3.4 Transmission Electron Microscopy of Viral-Like Particles [8, 9]

1. Place 100 µL 2% uranyl acetate into a droplet on parafilm.
2. Place another 100 µL molecular grade dH_2O into another droplet adjacent to the uranyl acetate droplet.
3. Place the Formvar-copper grid onto a clean area of the parafilm.
4. Pipette 10 µL of sample onto the copper side of the grid.
5. Allow the sample to adhere to the grid for 5 min.
6. Blot grid with filter paper.
7. Place grid sample side down on uranyl acetate droplet.
8. Allow sample grid to stain for 30 s.
9. Remove grid from uranyl acetate droplet and blot dry.
10. Place grid sample side down on dH_2O droplet.
11. Allow sample grid to wash for 30 s.
12. Remove grid from dH_2O droplet and blot dry.
13. Allow grid to air-dry for 5 min.
14. Visualize by transmission electron microscopy (>40,000×).

3.5 Viral DNA Extraction

1. Add 0.1 volume 2 M Tris–HCl pH 8.5/0.2 M EDTA to sample (e.g., add 50 µL to 500 µL sample).
2. Add 0.01 volume of 0.5 M EDTA to sample (e.g., add 5 µL to 500 µL sample).
3. Add 1 volume formamide (e.g., add 555 µL to 555 µL sample).
4. Add 1 µL glycogen (20 mg/mL) to each sample tube.

5. Incubate at room temperature for 30 min.
6. Split sample into two microcentrifuge tubes.
7. Add 2 volumes of room temperature 100% EtOH to each tube.
8. Centrifuge at 13,800 × *g* for 20 min to pellet.
9. Wash pellet 2× with 250 μL 70% EtOH.
10. Resuspend with 567 μL TE (10 mM Tris, 1 mM EDTA, pH 8.0).
11. *Optional stopping point: Store samples at −20 °C for up to 1 month.*
12. Add 30 μL 10% SDS.
13. Add 3 μL proteinase K (20 mg/mL).
14. Mix and incubate for 1 h at 37 °C.
15. Add 100 μL 5 M NaCl.
16. Add 80 μL CTAB/NaCl solution.
17. Mix and incubate at 65 °C for 10 min.
18. Add equal volume (~780 μL) of chloroform. Be sure to use chloroform in a chemical hood.
19. Mix and centrifuge at 13,800 × *g* for 10 min at room temperature.
20. Transfer top aqueous layer to a new microcentrifuge tube.
21. Add equal volume of 25:24:1 phenol:chloroform:isoamyl alcohol to supernatant. Be sure to use phenol:chloroform in a chemical hood.
22. Mix, and centrifuge at 13,800 × *g* for 10 min at room temperature.
23. Transfer top aqueous layer to a new microcentrifuge tube.
24. Add equal volume of chloroform to supernatant. Be sure to use chloroform in a chemical hood.
25. Mix, and centrifuge at 13,800 × *g* for 10 min at room temperature.
26. Transfer top aqueous layer to a new microcentrifuge tube.
27. Add 0.7 volumes isopropanol to supernatant.
28. Add 1 μL glycogen (20 mg/mL) to each sample tube.
29. Gently mix, and incubate at 4 °C overnight to precipitate DNA.
30. Centrifuge at 13,800 × *g* for 20 min at 4 °C.
31. Wash with cold 70% EtOH.
32. Allow air-drying for 15 min.
33. Resuspend with 50 μL molecular grade dH_2O.

34. Incubate for 5 min at 55 °C to promote resuspension.
35. Check DNA concentration using Qubit (Thermo Fisher Scientific) or spectrophotometer.
36. Check DNA quality using Agilent BioAnalyzer.
37. Check for host/bacterial contamination by 18S/16S PCR (Section 3.7).
38. Samples can be stored at 4 °C for 1–2 days, or at −20 °C for extended periods.

3.6 Viral RNA Extraction

1. Clean area with RNase-ZAP (or use 1% SDS solution) to ensure that working area is RNase free.
2. Split sample into another microcentrifuge tube (~250 μL per tube).
3. Add 3 volumes GITC buffer (e.g., add 750 μL GITC buffer to 250 μL sample).
4. Add 1 μL DTT (10 mM) to each sample tube.
5. *Optional stopping point: Store samples at −80 °C for up to 1 month.*
6. Add 0.2 volume chloroform (e.g., add 200 μL chloroform to 1 mL sample with GITC buffer), vortex, and incubate for 20 min at 4 °C.
7. Centrifuge at 13,800 × *g* for 20 min at 4 °C.
8. Transfer top aqueous layer into an RNase-free tube.
9. Add equal volume of isopropanol (e.g., add 500 μL isopropanol to 500 μL sample).
10. Add 1 μL glycogen (20 mg/mL).
11. Mix and incubate overnight at 4 °C to precipitate RNA.
12. Centrifuge at 13,800 × *g* for 20 min at 4 °C.
13. Wash with 250 μL cold RNase-free 70% EtOH.
14. Centrifuge at 13,800 x *g* for 5 min at 4 °C.
15. Repeat cold 70% EtOH wash.
16. Allow air-drying for 15 min.
17. Resuspend with 50 μL molecular grade dH_2O.
18. Incubate for 5 min at 55 °C to promote resuspension of RNA.
19. Check RNA concentration using Qubit or spectrophotometer.
20. Check quality of RNA using Agilent BioAnalyzer RNA Nano.
21. Check for host/bacterial contamination using 18S/16S PCR (Section 3.7).
22. Samples can be stored at −20 °C for 1–2 days, or at −80 °C for extended periods.

3.7 Quality Control to Determine Bacterial/Host Contamination

1. Standard PCR setup, per sample:
   (a) 2.5 µL PCR buffer (10×).
   (b) 1.0 µL BSA (1 mg/mL).
   (c) 1.0 µL $MgCl_2$ (10 mM).
   (d) 1.0 µL dNTPs (10 mM).
   (e) 1.0 µL Taq polymerase (10 U/µL).
   (f) 1.0 µL Primer 1 (1 mM).
   (g) 1.0 µL Primer 2 (1 mM).
   (h) 20 ng sample DNA.
   (i) Use positive vDNA control and negative control (no template) in separate sample reactions (*see* **Note 11**).
   (j) Fill to 25 µL with molecular grade dH_2O.
2. Touchdown PCR:
   (a) 94 °C for 5 min.
   (b) 94 °C for 30 s.
   (c) 65 °C for 1 m: −1 °C/cycle.
   (d) 72 °C for 2 min.
   (e) Repeat steps (b) to (d) 14 cycles.
   (f) 94 °C for 30 s.
   (g) 50 °C for 1 min.
   (h) 72 °C for 2 min.
   (i) Repeat steps (f–h) 14 cycles.
   (j) 72 °C for 10 min.
   (k) Hold at 4 °C until analysis.
   (l) Analyze for 18S/16S contamination by gel electrophoresis.

3.8 Viral RNA (vRNA) First-Strand Synthesis

1. Add 10 µL purified vRNA template.
2. Use a negative control of no vRNA template: Add 10 µL molecular grade dH_2O rather than template (*see* **Note 11**).
3. Use a positive control of known vRNA, if possible.
4. Add 4 µL 5× reverse transcriptase (RT) buffer.
5. Heat at 65 °C for 5 min to prime RNA.
6. Cool at 4 °C for 5 min.
7. Quick centrifugation spin to collect precipitation.
8. Primer mix setup, per sample:
   (a) 1.0 µL dNTPs (10 mM).
   (b) 1.0 µL N6 Primer (10 µM).
   (c) 1.0 µL dT18a primer (50 nM) (*see* **Note 12**).

9. Add 3 µL primer mix to each 14 µL sample.
10. Heat at 72 °C for 5 min to anneal primers to template.
11. Cool at 4 °C for 5 min.
12. Quick centrifugation spin to collect precipitation.
13. First Strand Master Mix Setup, per sample:
    (a) 0.5 µL DTT (10 mM).
    (b) 0.5 µL RNase inhibitor (40 U/µL).
    (c) 0.5 µL $MgCl_2$ (10 mM).
    (d) 0.5 µL DMSO.
    (e) 1.0 µL Reverse transcriptase (200 U/µL).
14. Add 3 µL first-strand master mix to each 17 µL sample.
15. Run 1st_Strand program on thermocycler for first-strand synthesis:
    (a) 25 °C for 10 min (to initiate reverse transcription).
    (b) 42 °C for 60 min.
    (c) 50 °C for 1 min.
    (d) 42 °C for 1 min.
    (e) Repeat steps (c–d) 9 cycles.
    (f) 65 °C for 20 min (to inactivate).
    (g) 4 °C for 5 min.
    (h) 4 °C Hold.
16. Quick centrifugation spin to collect precipitation.
17. *Optional stopping point: Store samples overnight at 4 °C.*

3.9 Viral Second-Strand Synthesis

1. Use 20 µL first-strand vRNA converted to cDNA.
2. Use a negative control of no cDNA template (*see* **Note 11**).
3. Use a positive control of cDNA template.
4. Second-strand synthesis reaction per reaction:
    (a) 4.0 µL Molecular grade dH_2O.
    (b) 3.0 µL 10× T4 DNA polymerase buffer.
    (c) 1.0 µL dUTPs (10 mM stock).
    (d) 0.5 µL RNase H (250 U/µL).
    (e) 0.5 µL DTT (10 mM stock).
    (f) 1.0 µL T4 DNA polymerase (150 U/µL).
5. Add 10 µL second-strand master mix to 20 µL sample.
6. Run 2nd_Strand program on thermocycler:
    (a) 4 °C for 2 min.
    (b) 16 °C for 90 min.

(c) 65 °C for 20 min (to inactivate DNA polymerase).

(d) 4 °C for 5 min.

(e) 4 °C Hold.

7. Quick centrifugation spin to collect precipitation.

3.10 DNA Cleanup

1. Allow SPRI beads to equilibrate to room temperature. Vortex to mix well.
2. Add equal volumes of sample and vortexed SPRI beads into microcentrifuge tube (e.g., add 30 μL SPRI beads to 30 μL sample).
3. Vortex briefly and incubate at room temperature for 5 min.
4. Place microcentrifuge tube on magnet holder and allow for beads to bind to magnet at the side of the tube for 5 min or longer, until bead pellet has formed on the magnet side of tube and solution has cleared.
5. Keep tube on magnet and remove all supernatant.
6. While leaving the tube on the magnet, wash twice with 250 μL freshly made 70% EtOH, and remove all liquid between washes.
7. Remove tube from magnet and allow beads to air-dry for 5 min, or until EtOH has fully evaporated.
8. Resuspend beads in 52.5 μL molecular grade H_2O, vortex, and place tube back on magnet. Allow beads to bind to magnet for 5 min or longer, until bead pellet has formed on the magnet side of tube and solution has cleared.
9. Transfer 50 μL of eluted sample from beads and place in a new microcentrifuge tube.

3.11 DNA Shearing

1. Transfer 52.5 μL DNA (vDNA and molecular grade dH_2O to 52.5 μL volume) to Covaris shearing tube.
2. Briefly spin to move sample to the bottom of the tube.
3. Turn on Covaris machine, open SonoLab program, and fill water bath to appropriate level with DI water.
4. Run DNA_Shear_45Sec program to shear DNA:
   (a) Covaris shearing settings:
   - 45 s
   - 50 W peak power
   - Duty factor 20%
   - 200 cycles/burst
   - Room temperature
5. Quick centrifugation spin to collect precipitation.

6. Transfer sheared DNA to PCR tube to continue with end repair.
7. Close SonoLab program, turn off Covaris machine, and clean and dry water bath.

3.12 End Repair

1. End-repair reaction setup, per sample reaction:
   (a) 6.0 μL 10× T4 DNA ligase buffer (contains ATP)
   (b) 2.0 μL Molecular grade dH_2O
   (c) 1.0 μL 10 mM dNTPs
   (d) 0.5 μL 5 U/μL T4 DNA polymerase
   (e) 0.5 μL 10 U/μL T4 polynucleotide kinase (PNK)
2. Add 10 μL of end-repair master mix to 50 μL sheared DNA.
3. Run End_Repair program on thermocycler:
   (a) 4 °C for 2 min
   (b) 20 °C for 30 min
   (c) 25 °C for 30 min
   (d) 4 °C for 5 min
   (e) 4 °C Hold
4. Add 60 μL SPRI beads. Vortex before use.
5. Repeat DNA cleanup steps (Section 3.10).
6. Elute with 50 μL molecular grade dH_2O.

3.13 Adenylate 3′ Ends

1. Adenylation reaction setup, per sample reaction:
   (a) 2.0 μL Molecular grade dH_2O
   (b) 6.0 μL 10× Klenow buffer
   (c) 1.0 μL 10 mM dATP
   (d) 1.0 μL 5 U/μL Klenow fragment DNA polymerase (3′-5′ exo-)
2. Add 10 μL of adenylation master mix to 50 μL end-repaired DNA.
3. Run DA_Tail program on thermocycler:
   (a) 4 °C for 2 min
   (b) 37 °C for 30 min
   (c) 4 °C Hold
4. Add 60 μL SPRI beads. Vortex before use.
5. Repeat DNA cleanup steps (Section 3.10).
6. Elute with 50 μL molecular grade dH_2O.

3.14 Adapter Ligation

1. Primary adapter ligation setup, per sample reaction:
   (a) 6.0 μL 10× T4 DNA ligase buffer (contains 10 mM ATP)

(b) 1.0 μL 10 μM Annealed indexed adapter (*see* **Note 13**)

(c) 0.5 μL 20 U/μL T4 DNA ligase

2. Add 6.5 μL of primary adapter ligation master mix to 50 μL 3′ adenylated DNA + 1 μL each annealed indexed adapter.
3. Run End_Repair program on thermocycler:
   (a) 4 °C for 2 min
   (b) 20 °C for 30 min
   (c) 25 °C for 30 min
   (d) 4 °C Hold
4. Secondary adapter ligation setup, per sample reaction:
   (a) 11.0 μL Molecular grade dH_2O
   (b) 1.0 μL T4 DNA ligase buffer (contains 10 mM ATP)
   (c) 0.5 μL 20 U/μL T4 DNA ligase
5. Add 12.5 μL of secondary adapter ligation master mix to 57.5 μL of primary adapter ligation.
6. Run Adap_Lig program on thermocycler:
   (a) 4 °C for 2 min
   (b) 16 °C for 2 h
   (c) 4 °C Hold
7. Add 70 μL SPRI beads. Vortex before use.
8. Repeat DNA cleanup steps (Section 3.10).
9. Elute with 50 μL molecular grade dH_2O.

3.15 Uridine Removal for Viral RNA Samples (Omit for Viral DNA Samples)

1. Add 5 μL UDG reaction buffer.
2. Add 0.5 μL UDG.
3. Incubate at 37 °C for 30 min.
4. Add 55 μL SPRI beads. Vortex before use.
5. Repeat DNA cleanup steps (Section 3.10).
6. Elute with 50 μL molecular grade dH_2O.

3.16 Size Selection of Adapter-Ligated Fragments

3.16.1 For a 550 bp Insert (for 300 bp Paired-End Sequencing)

1. For each sample, pipette 40 μL SPRI beads into a new microcentrifuge tube. Vortex before use.
2. Dilute beads with 40 μL molecular grade dH_2O.
3. Transfer 50 μL adapter-ligated sample into diluted SPRI bead centrifuge tube.
4. Vortex to mix.
5. Incubate at room temperature for 5 min.
6. Place microcentrifuge tube on magnet holder and allow for beads to bind to magnet at the side of the tube for 5 min or

longer, until bead pellet has formed on the magnet side of tube and solution has cleared.

7. Transfer supernatant to a new microcentrifuge tube. Discard tube with beads.
8. Add 30 μL undiluted and vortexed SPRI beads to supernatant tube.
9. Vortex to mix.
10. Incubate at room temperature for 5 min.
11. Place microcentrifuge tube on magnet holder and allow for beads to bind to magnet at the side of the tube for 5 min or longer, until bead pellet has formed on the magnet side of tube and solution has cleared.
12. Keep tube on magnet and remove all supernatant.
13. While leaving the tube on the magnet, wash twice with 250 μL freshly made 70% EtOH, and remove all liquid between washes.
14. Remove tube from magnet and allow beads to air-dry for 5 min, or until EtOH has fully evaporated.
15. Resuspend beads in 52.5 μL molecular grade H_2O, vortex, and place tube back on magnet. Allow beads to bind to the side of the tube for 5 min or longer, until bead pellet has formed on the magnet side of tube and solution has cleared.
16. Transfer 50 μL of eluted sample from beads and place in a new microcentrifuge tube.

3.16.2 For a 250 bp Insert (for 150 bp Paired-End Sequencing)

1. For each sample, pipette 55 μL SPRI beads into a new microcentrifuge tube. Vortex before use.
2. Dilute beads with 25 μL molecular grade dH_2O.
3. Transfer 50 μL adapter-ligated sample into diluted SPRI bead centrifuge tube.
4. Vortex to mix.
5. Incubate at room temperature for 5 min.
6. Place microcentrifuge tube on magnet holder and allow for beads to bind to magnet at the side of the tube for 5 min or longer, until bead pellet has formed on the magnet side of tube and solution has cleared.
7. Transfer supernatant to a new microcentrifuge tube. Discard tube with beads.
8. Add 30 μL undiluted and vortexed SPRI beads to supernatant tube.
9. Vortex to mix.

10. Incubate at room temperature for 5 min.
11. Place microcentrifuge tube on magnet holder and allow for beads to bind to magnet at the side of the tube for 5 min or longer, until bead pellet has formed on the magnet side of tube and solution has cleared.
12. Keep tube on magnet and remove all supernatant.
13. While leaving the tube on the magnet, wash twice with 250 µL freshly made 70% EtOH, and remove all liquid between washes.
14. Remove tube from magnet and allow beads to air-dry for 5 min, or until EtOH has fully evaporated.
15. Resuspend beads in 52.5 µL molecular grade H_2O, vortex, and place tube back on magnet. Allow beads to bind to the side of the tube for 5 min or longer, until bead pellet has formed on the magnet side of tube and solution has cleared.
16. Transfer 50 µL of eluted sample from beads and place in a new microcentrifuge tube.

3.17 Large-Scale PCR

1. PCR setup, per sample reaction:
   (a) 6.5 µL Molecular grade dH_2O
   (b) 5.0 µL 5× High-fidelity polymerase buffer
   (c) 1.0 µL 10 mM dNTPs
   (d) 1.0 µL 1 µM PCR Primer 1 (Illumina TruSeq P5)
   (e) 1.0 µL 1 µM PCR Primer 2 (Illumina TruSeq P7)
   (f) 0.5 µL 2 U/µL High-fidelity DNA polymerase
2. Add 15 µL of master mix to 10 µL adapter-ligated and size-selected DNA.
3. Run LargeScale program on thermocycler:
   (a) 95 °C for 5 min
   (b) 95 °C for 30 s
   (c) 60 °C for 60 s
   (d) 72 °C for 90 s
   (e) Repeat steps (b–d) using optimal # cycles (*see* **Note 14**)
   (f) 72 °C for 10 min
   (g) 4 °C Hold
4. Reconditioning PCR setup, per sample reaction (increases yield and decreases heteroduplex formation):
   (a) 11.5 µL Molecular grade dH_2O
   (b) 10.0 µL 5× High-fidelity polymerase buffer

(c) 1.0 μL 10 mM dNTPs
(d) 1.0 μL 1 μM PCR Primer 1 (Illumina TruSeq P5)
(e) 1.0 μL 1 μM PCR Primer 2 (Illumina TruSeq P7)
(f) 0.5 μL 2 U/μL Q5 High-fidelity DNA polymerase

5. Add 25 μL of master mix to 25 μL amplified DNA.
6. Run Recondition program on thermocycler:
   (a) 95 °C for 2 min
   (b) 95 °C for 30 s
   (c) 60 °C for 60 s
   (d) 72 °C for 90 s
   (e) Repeat steps (b–d) 3 cycles
   (f) 72 °C for 10 min
   (g) 4 °C Hold
7. Perform DNA cleanup steps (Section 3.10).
8. Elute in 55 μL molecular grade dH_2O.
9. Quantify by Qubit.
10. Quality control by BioAnalyzer.
11. Samples can be stored at 4 °C for 1–2 days, or at −20 °C for extended periods.

4 Notes

1. The term "bacteriophage" was originally used to describe entities that "ate" bacteria [14]. Hence, the Greek root meaning of "phage" is to eat, or bacteriophage, the "eater of bacteria." Prokaryotic viruses do not "eat" bacteria; they either lyse cells or integrate into genomes upon infection. Therefore, it is more accurate to term these viruses as what they infect, prokaryotic viruses.
2. Although the title of this chapter is for the isolation of bacteriophages from host-associated systems, you will also isolate eukaryotic viruses. This protocol is good for viral metagenome studies; simply substitute the word "virus" for "bacteriophage," and one will be able to obtain a full viral metagenome. If the goal is to select for prokaryotic viruses, it is recommended to use the optional CsCl step density gradient purification (Section 3.2).
3. Other buffers to consider using other than SM buffer are 1% potassium citrate (to 100 mL, add 1 g potassium citrate, 0.14 g Na_2HPO_4 $7H_2O$, and 0.024 g KH_2PO_4, pH to 7) and 10 mM sodium pyrophosphate (to 100 mL, add 0.27 g $Na_2P_2O_7$)

[15]. These buffers are supposed to help detach viruses from tissue. We have not had much success with these buffers, and have had the most success with SM buffer. These alternatives are included in case the user would like to try these buffers.

4. It is important to use small volumes in this protocol to help concentrate the sample and to work with less tubes throughout the procedure. Therefore, if one has large volumes of tissue slurry, it would be useful to precipitate the viruses into a smaller volume using pegylation [16]. Alternatively, Amicon filters (50 kD) can be used to concentrate viruses. Note that this will affect viral number counts, so if these counts are important, do not precipitate viruses until after sub-sampling for epifluorescence microscopy.
5. Chloroform is used at this step to lyse lipid membranes, particularly that of eukaryotic and prokaryotic cells. However, chloroform can also affect (and lyse) enveloped viruses. It is not recommended to use chloroform at this step because it can affect enveloped viruses while increasing the amount of host and bacterial DNA in the sample. Rather, an additional 0.45 μm filtration step can be used to remove eukaryotic and prokaryotic cells while preserving the enveloped viruses. This step is included as optional for those wishing to use this step.
6. Free DNA is common in host-associated samples. Further, released DNA through filtration of cells (unlikely) or chloroform lysis of cells (most likely) can obscure viral DNA isolation later in the protocol. It cannot be stressed enough how important it is to DNase your samples, as it affects viral number counts (*see* **Note 10**), and can affect what is sequenced. If host or bacterial contamination is observed, a second DNase step should be added during the isolation of viruses in future experiments.
7. It is optional to treat the sample with RNase. RNase is useful to remove prevalent rRNAs from a host-associated sample (>80% of the sample). This is particularly necessary when isolating RNA viruses. However, it is difficult to remove RNases from a sample once added. They are not heat-inactivated. It is necessary to use DTT or 2-mercaptoethanol to inactivate the RNases prior to RNA isolation.
8. Heat inactivation works for DNase, but not for RNase. It is necessary to use DTT or 2-mercaptoethanol to inactivate the RNases prior to RNA isolation.
9. After removal of sample with needle, the hole left behind will cause the rest of the gradient to come out of the tube, so have another waste container available to collect it.
10. The enumeration of viruses is rife with pitfalls. Often, viral-like particle (VLP) counts are overestimated in host-associated

systems due to not eliminating host-generated factors (such as vesicles and free DNA) [17]. Therefore, it is important that VLP counts are conducted after filtration, and importantly after DNase treatment to eliminate host factors. When enumerated properly, the VLP numbers are usually equivalent to bacterial numbers in host-associated systems. Please refer to the work by Ortmann and Suttle for reference [7].

11. It is of vital importance to use both positive and negative controls throughout the random amplification library preparation and during the PCRs. A positive control is needed to ensure that your protocol is working throughout. More importantly, a negative control is needed to ensure that contaminating DNA is not infiltrating your protocol and potentially sequenced as a false positive. These controls are often erroneously omitted from the protocol, but if not included false outcomes can creep into your sequencing results.
12. The VN-anchored oligo dT primer allows the primer to only anneal to the 5′ end of the poly(A) tail of mRNA, allowing for more efficient cDNA synthesis.
13. Excess adapters can interfere with sequencing [18]. The adapters have to be diluted relative to the starting material. Using 10 μM adapter stock, for samples >100 ng, do not dilute. For samples 10–100 ng, make a 1:10 dilution of 10 μM adapter stock to 1 μM. For samples 1–10 ng, make a 1:20 dilution, or 500 nM. For samples <1 ng, make a 1:30 dilution, or 250 nM. Add 1 μL of these diluted adapters to each reaction.
14. Use the minimum number of cycles to barcode and amplify the amount of DNA needed to sequence [19]. One should ensure that each fragment of DNA has an adapter on it for sequencing. To achieve this, use the minimum number of cycles of 8, so if starting with more than 1 ng of DNA, use 8 cycles. If one has 1 ng of DNA or less, use 12 cycles. If one has 1 pg of DNA or less, use 21 cycles. Keep in mind that amplifying with too many cycles can lead to amplification bias and sequencing artifacts, so use the minimum number of cycles to obtain the amount of DNA needed to sequence.

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